Oral treatment of rodents with insecticides for control of sand flies (Diptera: Psychodidae) and the fluorescent tracer technique (FTT) as a tool to evaluate potential sand fly control methods

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ABSTRACT: In laboratory studies, insecticides (diflubenzuron, novaluron, methoprene and, pyriproxyfen) that have been incorporated into rodent diets were effective as feed-throughs against sand fly larvae. Novaluron also was effective against sand fly larvae at low concentrations and under simulated field conditions. Ivermectin has been shown to be effective as a systemic insecticide, killing 100% of blood-feeding sand flies for up to seven d after rodents were treated. The fluorescent tracer technique (FTT) is the use of certain fluorescent dyes (rhodamine B or uranine O) as feed-through transtadial biomarkers for phlebotomine sand flies, systemic biomarkers for blood-feeding sand flies, and permanent markers for nectar-feeding sand flies. The results of these laboratory studies provide proof of concept for the FTT and indicate that the FTT could be used to delineate specific foci with rodent/sand fly associations that would be susceptible to control by using feed-through or systemic insecticides, or foci where insecticide-treated sugar baits could be used against sand flies. ***Journal of Vector Ecology* 36 (Supplement 1): S132-S137. 2011.**

Keyword Index: Feed-through, systemic insecticide, sand fly, *Phlebotomus papatasi*, control.

INTRODUCTION

Phlebotomine sand flies are major biting pests of man and are the vectors of several viruses, the bacterium *Bartonella bacilliformis*, and, most importantly, the protozoan parasites that cause leishmaniasis. The World Health Organization considers leishmaniasis to be an emerging and uncontrolled disease. According to the World Health Organization (2010), there has been a marked increase in the number of recorded cases of leishmaniasis in the past ten years. Currently, there are an estimated two million new cases annually and about 12 million people are infected. About 75% of the new cases are cutaneous leishmaniasis and 25% are visceral leishmaniasis. The majority of the cases of leishmaniasis are found among the poorest people of the world, and factors such as poor housing and displacement are among the factors that contribute to this phenomenon. Throughout North Africa, the Middle East, and southwest Asia, *Phlebotomus papatasi* is the primary vector of *Leishmania major*, the causative agent of zoonotic cutaneous leishmaniasis (ZCL). *Phlebotomus duboscqi* is the primary vector of *L. major* in ZCL foci in Sub-Saharan Africa.

In arid and semi-arid areas, *P. papatasi* and *P. duboscqi* exhibit a close association with many species of burrowing rodents that are reservoirs of *L. major*. While adults and larvae of *P. papatasi* have been recovered from several different sources around areas of human habitation, adult and immature *P. papatasi* are recovered almost exclusively from rodent burrows in less-developed areas or natural habitats (Artemiev et al. 1971, Doha et al. 1990, Desjeux 1991). In Kenya, adults and larvae of *P. duboscqi* have been recovered from the burrows of rodent reservoirs of *L. major* (Mutero et al. 1991).

Adult female sand flies require nutrients from blood for reproduction, and by sharing a burrow with rodents they have continuous access to a source of blood. The burrows of rodent reservoirs of *L. major* are often located in close proximity to vegetation, which can serve as a convenient food source for the rodents, and adult sand flies can also benefit from the proximity to plants, from which they obtain sugar meals (Schlein and Warburg 1986). Sand fly larvae also have been observed feeding on the feces of rodents inside of a burrow (WHO 1968). Both adult and immature sand flies benefit from the microhabitat created within rodent burrows. Rodents in arid and semi-arid areas construct burrows as refuges from the high diurnal temperatures, creating a habitat that has a moderate temperature and high relative humidity (Grenot 2001, Shenbrot et al. 2002).

Control of sand flies in ZCL foci using insecticide treatments has not been successful because of the difficulty of delivering insecticides to the precise microhabitats of immature and adult sand flies at the ends of long and complex tunnels inside rodent burrows (Karapet'ian et al. 1983). Additionally, even treatments that are initially effective are short-lived, and frequent reapplication of insecticides would be necessary (Karapet'ian et al. 1983).

The purpose of this article is to present the findings of

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14. ABSTRACT

In laboratory studies, insecticides (diflubenzuron, novaluron, methoprene and, pyriproxyfen) that have been incorporated into rodent diets were effective as feed-throughs against sand fly larvae. Novaluron also was effective against sand fly larvae at low concentrations and under simulated field conditions. Ivermectin has been shown to be effective as a systemic insecticide, killing 100% of blood-feeding sand flies for up to seven d after rodents were treated. The fluorescent tracer technique (FTT) is the use of certain fluorescent dyes (rhodamine B or uranine O) as feed-through transtadial biomarkers for phlebotomine sand flies, systemic biomarkers for blood-feeding sand flies, and permanent markers for nectar-feeding sand flies. The results of these laboratory studies provide proof of concept for the FTT and indicate that the FTT could be used to delineate specific foci with rodent/sand fly associations that would be susceptible to control by using feed-through or systemic insecticides, or foci where insecticide-treated sugar baits could be used against sand flies. Journal of Vector Ecology 36 (Supplement 1): S132-S137. 2011.

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a promising line of research into methods for the control of sand flies that take advantage of the close ecological interaction between sand flies and rodents in Old World ZCL foci using feed-through or systemic insecticides incorporated into rodent baits. Additionally, the fluorescent tracer technique (FTT), a tool using a fluorescent dye as a systemic or feed-through transtadial biomarkers for sand flies, is presented. The use of the FTT is discussed in the context of these novel control methods.

RODENT FEED-THROUGH

Laboratory proof of concept has been established for rodent feed-through control of sand fly larvae using five different insecticides: diflubenzuron, novaluron, methoprene, pyriproxyfen, and ivermectin. These compounds were selected for evaluation as rodent feed-throughs because of their low mammalian toxicity and because the majority of each compound is eliminated in the feces of orally-dosed mammals (FAO 1981, Campbell et al. 1983, FAO 2005). To evaluate the laboratory efficacy of these rodent feed-through insecticides, at least three concentrations of each insecticide were tested (Table 1). Syrian hamsters (*Mesocricetus auratus*) and *Phlebotomus papatasi* were the rodent and sand fly models used in these studies, respectively. The rodent feeding protocol and sand fly larvae bioassay are described in detail in Mascari et al. (2007a). In brief, technical formulations of the insecticides were added to laboratory rodent chow, and hamsters were fed insecticide-treated diets for nine days. The amount of food consumed by the hamsters was recorded each day to determine if any of the concentrations of insecticide affected the palatability of the food for the hamsters. The feces of the hamsters were collected and fed to 2nd instar larvae, and the development and survival of the sand flies were recorded.

None of the concentrations of diflubenzuron, novaluron, methoprene, or pyriproxyfen tested in these studies significantly affected the amount of food consumed by the hamsters. The amount of food eaten by hamsters fed a diet containing 2, 6, 10, or 20 mg/kg ivermectin was not significantly different from hamsters fed an untreated diet, but hamsters that were fed a diet containing 40, 60, or 100 mg/kg ivermectin ate significantly less food than hamsters fed an untreated diet.

The two chitin synthesis inhibitors diflubenzuron and novaluron evaluated in these studies were effective as rodent feed-through insecticides against sand flies at all of the concentrations tested (Mascari et al. 2007a, 2007b). All sand fly larvae that consumed feces of hamsters that had been fed a diet containing as little as 8.97 mg/kg diflubenzuron or 9.88 mg/kg novaluron died before pupation. Larvae that consumed feces of diflubenzuron-treated hamsters died during the larva-to-pupa molt, while larvae fed feces of novaluron-treated hamsters died during larval molts.

Of the two juvenile hormone analogs evaluated in these studies, pyriproxyfen was effective against sand fly larvae when incorporated at lower concentrations into hamster food compared to methoprene (Mascari et al. 2011). Feces of hamsters fed a diet containing 9.82 mg/kg pyriproxyfen or more prevented all sand fly larvae that fed on it from successfully pupating. The feces of methoprene-treated hamsters greatly reduced the percentage of larvae that pupated and prevented all sand flies from emerging as adults at all concentrations tested except 9.79 mg/kg. The observation that methoprene were less effective than similar concentrations of pyriproxyfen is consistent with the literature on the effects of the two juvenile hormone analogs on mosquito larvae (Ali et al. 1995, Nayar et al. 2002).

A total of six diet concentrations of ivermectin in rodent food ranging from 2 to 100 mg/kg were evaluated as feed-throughs against sand fly larvae (Mascari et al. 2008). Feces of hamsters fed the lower concentrations of ivermectin caused significant, but not 100%, mortality when fed to sand fly larvae. Feces of hamsters fed a diet containing 20, 60, or 100 mg/kg ivermectin killed all sand fly larvae that fed on it. In these bioassays, the sand fly larvae died, on average, within about four days after feeding on the feces of ivermectin-treated hamsters. While ivermectin was found to be less than 100% effective against sand fly larvae when incorporated into rodent food at low concentrations and unpalatabale to hamsters at higher concentrations, a diet concentration of 20 mg/kg was found to be both effective against sand fly larvae and palatable to hamsters. In contrast to the chitin synthesis inhibitors and juvenile hormone analogs evaluated in these studies, which affected sand fly larvae at certain stages of their development, ivermectin

Table 1. Summary of effective concentrations of rodent feed-through insecticides and their effects against immature sand flies.

Insecticide	Effective bait concentrations (mg/kg)	Effects
Chitin synthesis inhibitor		
Diflubenzuron	8.97, 89.7, 897	Mortality at larva-to-pupa molt
Novaluron	9.88, 98.8, 988	Mortality at larval molt
Juvenile hormone analog		
Methoprene	97.9, 979	Mortality at 4th instar or pupal stage
Pyriproxyfen	9.82, 98.2, 982	Mortality at 4th instar
Macrocyclic lactone		
Ivermectin	20	Mortality after 3-5 days

induced an acute response in the sand fly larvae, killing them within a few days after exposure to feces of ivermectin-treated hamsters.

Effective concentrations were identified for each insecticide that was tested in these studies, and the results of these studies provide proof of concept for the control of sand fly larvae using these insecticides as rodent feed-throughs in the laboratory. Further evaluation of the performance of novaluron as a rodent feed-through has been conducted under simulated field conditions, and it appears that novaluron is a promising candidate for further evaluation in field studies (Mascari and Foil 2010a). Laboratory studies demonstrated that feces of novaluron-treated hamsters that had been aged for 150 days under conditions simulating the inside of a rodent burrow (high heat and humidity) still killed over 80% of sand fly larvae that fed on the feces. Also in this study, novaluron was shown to be effective as a feed-through against sand fly larvae when novaluron-treated food made up only a small portion of a hamster's daily diet.

Whether feed-through control of sand fly larvae is effective in a particular ZCL focus is dependent on whether the sand fly larvae in that location feed on the feces of the rodents targeted with baits. Because many different rodent-sand fly interactions exist, it is essential to demonstrate this fact before, or simultaneous with, field evaluations of insecticide-treated rodent baits. Unfortunately, it is impractical to directly observe sand fly larvae feeding on rodent feces in nature, and no alternative methods currently are available to demonstrate what sand fly larvae are feeding on. Furthermore, since larval sampling cannot be used to demonstrate the success or failure of feed-through sand fly control, comparisons between the number of adult sand flies collected at treated and control sites would be the only available method. Using adult sampling to demonstrate population reductions due to larval control have inherent problems such as failure to detect larval control because of immigration of adult sand flies from outside the study area, or observing a reduction in the adult population caused by natural population decline and incorrectly attributing it to successful larval control.

Confronted with these issues surrounding the application and evaluation of rodent feed-through control of sand flies, the development of the FTT could be a useful tool in the field (Mascari and Foil 2009). In laboratory studies, rhodamine B (a fluorescent dye with a high quantum yield and low mammalian toxicity that is eliminated in the feces of orally-dosed mammals) incorporated into rodent bait marks rodents that consume the bait for more than three months. The feces of the rodents fed rhodamine B-treated food were fluorescent when observed under a fluorescence microscope with the proper filter. Most importantly, adult male and female sand flies that fed as larvae on the feces of rhodamine B-treated rodents were fluorescent when examined under fluorescence microscopy. Sand fly larvae fed feces of rhodamine B-treated rodents developed at the same rate as control sand flies, their survival to adult emergence was not significantly different from that of control sand flies, and the adult sand flies remained marked with rhodamine B for life.

In the context of field evaluations of rodent feed-through insecticides for control of sand flies, essential information could be obtained through the incorporation of rhodamine B into rodent baits. The fact that rhodamine B marks bait-fed rodents for several months could help monitor the consumption of baits by rodents targeted in field trials. Since the collection of adult sand flies that are marked by rhodamine B would demonstrate that sand fly larvae are feeding on the feces of bait-fed rodents, one could then identify whether sand flies in a certain rodent-sand fly interaction feed on the feces of rodents and therefore could be targeted with feed-through insecticides. Furthermore, The FTT would allow the estimation of the proportion of sand flies in a population that could be eliminated using rodent feed-through insecticides. Finally, the FTT would allow an evaluation of the effects of rodent baits containing feed-through insecticides on sand fly populations. This could be achieved by treated some sites with rodent baits containing rhodamine B plus a feed-through insecticide and other sites with rodent baits containing rhodamine B alone. Successful control of sand flies using rodent feed-through insecticides could be demonstrated through the collection of rhodamine B-marked sand flies at sites treated with rodent baits treated with rhodamine B alone, and the absence of rhodamine B-marked sand flies at sites treated with rodent baits containing rhodamine B plus a feed-through insecticide.

SYSTEMIC

Ivermectin was effective as a rodent feed-through insecticide against sand fly larvae, and it also was evaluated as a systemic insecticide against blood-feeding adult sand flies (Mascari and Foil 2010b). In the laboratory, a diet containing 20 mg/kg was fed to hamsters for nine days. The hamsters were anesthetized and sand flies (*P. papatasi*) were allowed to take blood meals from ivermectin-treated hamsters 0, 3, 7, and 14 days after they were withdrawn from ivermectin-treated diets. For sand flies that took blood meals from ivermectin-treated hamsters 0, 3, or 7 days post-treatment, 100% mortality was observed. Mortality of sand flies that took blood meals from hamsters 14 days post-treatment was not significantly different from the morality of sand flies that took blood meals from untreated hamsters.

This study demonstrates proof of concept of oral treatment of rodents with ivermectin for the control of adult female sand flies. The finding that ivermectin treatment of rodents is effective against sand flies for seven days but becomes ineffective by 14 days post-treatment could direct the frequency with which baiting must occur in the field to have a sustained effect on a sand fly population (baiting does not need to be continuous but should be conducted weekly).

The FTT also could be used in conjunction with rodent baits containing a systemic insecticide like ivermectin. In laboratory studies, hamsters were fed a diet containing rhodamine B, and adult female sand flies were allowed to

take bloodmeals from these hamsters. Sand flies that took a bloodmeal from a rhodamine B-treated hamster were fluorescent when examined under fluorescence microscopy and could be distinguished from sand flies that took a bloodmeal from an untreated hamster.

As with rodent baits containing a feed-through insecticide, important data could be obtained through the incorporation of rhodamine B into rodent baits containing a systemic insecticide. The extent to which sand flies in certain rodent-sand fly interactions take bloodmeals from rodents targeted with baits could be determined, and rodent-sand fly interactions that could be targeted with baits containing a systemic insecticide could be identified. The use of rodent baits containing rhodamine B also could allow the evaluation of the effect of rodent baits containing a systemic insecticide on sand fly populations. Using an experimental design similar to that discussed for a field evaluation of feed-through insecticides, some sites would be treated with rodent baits containing rhodamine B plus a systemic insecticide and other sites with rodent baits containing rhodamine B alone. Successful control of sand flies using systemic insecticides could be demonstrated through the collection of rhodamine B-marked bloodfed female sand flies at sites treated with rodent baits treated with rhodamine B alone and the absence of rhodamine B-marked sand flies at sites treated with rodent baits containing rhodamine B plus a systemic insecticide.

Preliminary analysis of sand flies collected during a six-month field trial of rhodamine B-treated rodent baits in Baringo District, Kenya, in 2009 revealed that approximately half of all bloodfed female sand flies collected were marked with rhodamine B (unpublished data). These results suggest that a large proportion of sand flies in this location feed on baited rodents and that systemic insecticides could be an appropriate control measure against sand flies in Kenya. An evaluation of rodent baits containing ivermectin currently is being conducted at this same location.

SUGAR

Adult males and females of *P. papatasi* feed on naturally occurring sugars such as aphid honeydew and plant nectars (Cameron et al. 1995, Schlein and Muller 1995). In a study in an arid habitat in Israel, a high proportion of sand flies have been shown to feed on sugar solutions containing food dyes sprayed on plants, based on the large number of food dye-marked sand flies collected (Schlein 1987). However, laboratory studies have shown that the food dyes carmoisine and indigotine do not persist in the sand flies for very long (fewer than 20% were marked after only three days), and it is sometimes difficult to distinguish sand flies marked by these food dyes from unmarked sand flies (Mascari and Foil 2010c). The use of these dyes in the field is also limited by the fact that only a single dye (and therefore treatment) can be evaluated at a site because different food dyes cannot be used in combination; the red mark from carmoisine cannot be detected in a sand fly also marked with indigotine.

The fluorescent dyes rhodamine B and uranine O are potential alternatives to these food dyes for incorporation into sugar solution for field studies. Like the food dyes carmoisine and indigotine, it has been shown in laboratory studies that rhodamine B and uranine O do not affect the survival of sand flies that have fed on sugar solution containing these dyes (Mascari and Foil 2010c). Also in these studies, sand flies that have fed on sugar solution containing as little as 10 mg/L rhodamine B or uranine O could be distinguished from sand flies that fed on an untreated sugar solution using fluorescence microscopy. However, unlike food dyes, rhodamine B and uranine O could be detected in dye-treated sand flies for the remainder of the life of the sand fly. Both rhodamine B and uranine O could be detected by fluorescence microscopy in sand flies that fed on sucrose solution containing both dyes, or when the dyes were fed sequentially to sand flies.

The use of fluorescent dyes in sugar solution could allow the identification of sand flies that feed on sugar baits sprayed on vegetation in the field. Using this technique, the proportion of a sand fly population that could be targeted with insecticide-treated sugar baits could be identified and the effectiveness of insecticide-treated sugar baits could be evaluated. Differentially marking sand flies by spraying two solutions containing rhodamine B or uranine O could be used to measure the relative attractiveness of different sugar baits to sand flies.

DISCUSSION

Survival of sand fly larvae was greatly reduced when they were fed feces of hamsters that had been fed chitin synthesis inhibitors, juvenile hormone analogues, or ivermectin. Diflubenzuron appears to have a specific effect on the pupation of sand flies, consistent with findings for larvae of house flies and stable flies treated with diflubenzuron that also died in the larva to pupa molt (Wright 1974). Sand fly larvae that were fed feces of hamsters treated with novaluron died, while control sand flies molted from 2nd to 3rd instar larvae. Novaluron has been shown to have a similar effect on spined soldier bugs, which die as larvae after being treated with novaluron (Cutler et al. 2006). Sand flies fed feces of hamsters treated with juvenile hormone analogues had a prolonged larval stage and most died as late instar larvae or as larva-pupa intermediates, which is consistent with their mode of action. Ivermectin was effective as a feed-through at a concentration that is palatable to hamsters. We have also demonstrated that feeding hamsters a diet containing 20 mg/kg ivermectin yielded 100% control of adult female sand flies that took blood meals from these hamsters for up to seven days after they were withdrawn from their diets. This study confirms that ivermectin-treated rodent baits developed as a feed-through to control sand fly larvae could also have a collateral effect on the survival of blood-feeding sand flies.

Significant control of sand fly larvae was observed when they were fed feces of novaluron-treated hamsters that had been aged for up to 150 days. This suggests that novaluron could persist in the larval habitat and remain active against

sand fly larvae for a long period of time. We also observed that the feces of hamsters fed novaluron-treated food as a portion of their daily diet were equally as effective against sand fly larvae as feces of hamsters exclusively fed a novaluron-treated diet. The results of this experiment suggest that when novaluron is eliminated by orally-dosed hamsters, it is uniformly distributed in the feces. This is an important observation because artificial baits for wildlife do not fully supplant naturally available food sources. The results of the experiment indicate that novaluron would be effective under circumstances where baits make up only a small portion of the diet of the target rodents in a field setting.

These studies constitute proof of concept for feed-through control of sand fly larvae using rodents with three different classes of insecticides. We also found that ivermectin was effective as a systemic insecticide against blood-feeding female sand flies. This is an important first step in future development of rodent baits containing feed-through or systemic insecticides. We have also found that novaluron is a particularly good candidate for further evaluation as a feed-through against sand fly larvae. Since the results of this study suggest that novaluron could be effective as a rodent feed-through insecticide in a field setting, the next step would be to evaluate the effects of novaluron-treated baits on sand fly populations in different rodent/sand fly associations.

The FTT was developed to establish for different rodent-sand fly interactions the extent to which sand fly larvae feed on feces and adult female sand flies feed on blood of bait-fed rodents. In the laboratory, rodents and their feces have been marked using rhodamine B-treated bait. Sand fly larvae that have fed on feces of rhodamine B-treated rodents and adult females that have taken a blood meal from rhodamine B-treated rodents are fluorescent when examined under fluorescence microscopy. Additionally, the use of the FTT in conjunction with sugar baits has been evaluated in the lab, and rhodamine B and uranine O have both been shown to be non-toxic to sand flies and mark sand flies for life. The dyes also can be used in combination, allowing for more sophisticated field studies that evaluate multiple treatments.

Because it is unlikely that all sand flies in an area could be eliminated using a single control method, the FTT could be used to identify which insecticide application, or combination of applications, would be most effective against a sand fly population. The FTT could allow a researcher to determine the extent to which sand fly populations would be susceptible to control using rodent feed-through insecticides, systemic insecticides, or insecticide-treated sugar baits, but equally as important, in the event that an insecticide treatment isn't effective, the FTT could be used to establish reasons why and to plan future research accordingly.

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